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Symbiotic characteristics of *Bradyrhizobium diazoefficiens* USDA 110 mutants associated with shrubby sophora (*Sophora flavescens*) and soybean (*Glycine max*)

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ABSTRACT

Site-specific insertion plasmid pVO155 was used to knockout the genes involved in the alternation of host range of strain *Bradyrhizobium diazoefficiens* USDA 110 from its original determinate-nodule-forming host soybean (*Glycine max*), to promiscuous and indeterminate-nodule-forming shrubby legume sophora (*Sophora flavescens*). Symbiotic phenotypes of these mutants inoculated to these two legumes, were compared to those infected by wild-type strain USDA 110. Six genes of the total fourteen Tn*5* transposon mutated genes were broken using the pVO155 plasmid. Both Tn*5* and pVO155-inserted mutants could nodulate *S. flavescens* with different morphologies of low-efficient indeterminate nodules. One to several rod or irregular bacteroids, containing different contents of poly-β-hydroxybutyrate or polyphosphate were found within the symbiosomes in nodulated cells of *S. flavescens* infected by the pVO155-inserted mutants. Moreover, none of bacteroids were observed in the pseudonodules of *S. flavescens*, infected by wild-type strain USDA 110. These mutants had the nodulation ability with soybean but the symbiotic efficiency reduced to diverse extents. These findings enlighten the complicated interactions between rhizobia and legumes, i. e., mutation of genes involved in metabolic pathways, transporters, chemotaxis and mobility could alter the rhizobial entry and development of the bacteroid inside the nodules of a new host legume.

1. Introduction

Sophora flavescens, the shrubby sophora (“Ku Shen” in Chinese), is a medicinal leguminous herb, whose roots secrete nitrogen-containing sophocarpidine or matrine, which could be used as insecticide or anti-hepatoma agents. This legume can establish effective symbioses with various rhizobia belonging to different genera and cross-nodulation groups (Jiao et al., 2015) including with *Sinorhizobium fredii* but not with *Bradyrhizobium diazoefficiens*, two well-known soybean-nodulating rhizobia (Zhang et al., 2011; Tian et al., 2012; Delamuta et al., 2013). Although the identical structures of lipochitooligosaccharides (LCOs) secreted by these two rhizobial species may endow nodulation on the common host, soybean (*Glycine max*), they still have their distinct symbiotic partners. Comparatively, *S. fredii* presents broader host ranges forming either indeterminate nodules on Chinese liquorice (*Glycyrrhiza uralensis*) (Crespo-Rivas et al., 2016), pigeonpea (*Cajanus cajan*) (Li et al., 2015) and shrubby sophora (*S. flavescens*) (Jiao et al., 2015); or determinate nodules on wild soybean (*Glycine soja*) (Scholla and Elkan, 1984; Wu et al., 2011), cowpea (*Vigna unguiculata*) (Scholla and Elkan, 1984), bird's-foot trefoils (*Lotus burttii*) (Acosta-Jurado et al., 2016) as well as ineffective symbiosis with purple bush-bean (*Macroptilium atropurpureum*), phasey bean (*Macroptilium lathyroides*), prickly sesban (*Sesbania cannahina*), mung bean (*Vigna radiata*), and different cultivars of soybean (Keyser et al., 1982). While *B. diazoefficiens* is specific for purple bush-bean, cowpea and mung bean (Göttfert et al., 1990; Lardi et al., 2016) besides soybean as its limited partners usually forming determinate nodules.

Affiliated to *Bradyrhizobium japonicum*, wild-type strain USDA 110 was originally isolated from root nodules of soybean grown in Florida, USA (Mathis et al., 1997) and was renamed as *B. diazoefficiens* by

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Delamuta et al. (2013). Strain USDA 110 is one of the most agriculturally important N_2-fixing bacterium associated with soybean (Narozna et al., 2015) and it has been widely employed as a type strain in various studies of genetics, genome (Kaneko et al., 2002), and molecular interaction and host specificity with soybean (van Berkum and Fuhrmann, 2009; Quelas et al., 2010; Ledermann et al., 2015). Previous reports have suggested that soybean controls the specific nodulation with *Bradyrhizobium* or *Sinorhizobium* by specific genes (Yang et al., 2010; Fan et al., 2017), while several genes in rhizobia also compete the soybean cultivar compatibility (Bellato et al., 1997; Annapurna and Krishnan, 2003). In our most recent study (Liu et al., 2018), symbiotic promiscuity between *S. flavescens* and six different rhizobia was explored molecularly, and the functional nodules, formed on *S. flavescens* by Tn*5* transposon mutagenesis of strain USDA 110, were reported. However, the genetic characterization of the mutated genes as well as the symbiotic properties of these Tn*5* mutants in interacting with *S. flavescens* and soybean have not been studied in details. In addition, whether the pseudonodules induced by the wild-type USDA 110 and the indeterminate nodules induced by the mutants contained bacteroids or not are still not known.

The aim of present study was to identify the mutated genes and analyze the symbiotic characteristics of the mutants of *B. diazoefficiens* on *S. flavescens*, and soybean, as well as to explore the potential factor (s) causing negative response from the host plant.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains, Tn*5* mutants, and plasmids used in this study are listed in Supplementary Table S1. *B. diazoefficiens* USDA 110 was cultured at 28 °C on TY medium (Beringer, 1974) or yeast extract mannitol (YEM) medium (Vincent, 1970) supplemented with 10 μg mL^{-1} trimethoprim (TMP). *Escherichia coli* DH5α was grown at 37 °C in Luria–Bertani (LB) medium (Miller, 1972) supplemented with 50 μg mL^{-1} kanamycin (Km).

2.2. Identification of Tn5 inserted genes using pVO155 plasmid

The construction of Tn*5* mutants was described previously (Liu et al., 2018). To verify the symbiotic phenotypes caused by Tn*5* transposon insertion, six genes encoding for phosphoenolpyruvate (PEP) synthase, hypothetical membrane protein, flagellar hook-basal body protein, NAD(P)H-quinone oxidoreductase, SH3 domain-containing protein and magnesium transporter, which were defined as BDT-2, BDT-4, BDT-7, BDT-10, BDT-18 and BDT-28, respectively, were mutated via single-crossover recombination using site-specific insertion plasmid pVO155 (Table 1 and Supplementary Table S1). To knockout these six genes, internal regions with different lengths of them were amplified respectively using corresponding primer pairs and PCR annealing conditions listed in Supplementary Table S2. Then, the obtained PCR product was ligated into digested pVO155 plasmid with *Xba*I and *BamH*I restriction endonucleases according to the manufacturer's instructions of seamless clone kit (No. C5891, Clone Smarter Technologies Co.). The recombined plasmid was transformed into heat-shocked *E. coli* DH5α strains and triparentally conjugated with the wild-type strain USDA 110 and the helper strain DH5α containing pRK2013 (Km^r) plasmid. The recombined conjugants were selected on TY agar plates containing two antibiotics of Km (50 μg mL^{-1}) and TMP (10 μg mL^{-1}) simultaneously. Genetic modification in the single crossover was verified by PCR amplification, using the corresponding detection primer and universal primer M13R, listed in Supplementary Table S2, and DNA sequencing by Tsingke Biological Technology Co. Ltd. Sequence analyses and comparison were carried out referring to the whole genome of strain USDA 110 (GenBank No. BA000040.2) and related bacterial sequence data in GenBank database.

2.3. Nodulation characterization of wild-type strain and mutants

Surface sterilization and germination of *S. flavescens* (cultivar Zhenku No. 2) and soybean (cultivar Jidou No. 17) seeds were performed as reported by Jiao et al. (2015). Seedlings were transferred to a Leonard jar assembly containing sterile vermiculite moistened with low-nitrogen plant nutrient solution (Vincent, 1970). Each treatment consisted of 20 plants, with one plant per jar. Each plant was inoculated with 1 mL of rhizobial or their Tn*5* or pVO155 mutant culture (concentration of OD_{600} = 0.2, diluted with 0.8% (w/v) NaCl). Nodulation test was repeated three times. Plants were grown in a greenhouse under a 16-h light (25 °C)/8-h dark (16 °C) photoperiod, and were harvested for checking the phenotype from 26 to 45 days post-inoculation (dpi). Biological characteristics, including nodule number, fresh nodule weight, shoot dry weight and chlorophyll content, were determined and compared among plants inoculated with wild-type strain and the mutants. Leaf chlorophyll concentration was determined with a SPAD-502 plus Meter (Konica Minolta, Osaka, Japan) (Ling et al., 2011). Plant shoots were dried at 65 °C for 5 days and then weighed. The number of root nodules per plant was counted. To visualize the morphology of bacteroids inside nodules, paraffin section or ultrathin sections of nodules were prepared and observed under a light microscope or transmission electron microscope (TEM) (Model: JEM-1230, Tokyo, Japan) as described previously (Li et al., 2013).

2.4. Motility assay

Wild-type strain USDA 110 and BDT-7pvo mutant (the *flgE* gene encoding for flagellar hook-basal body protein was knocked out) were grown in the TY broth (Beringer, 1974) to mid-log phase. The cell optical densities at 600 nm (OD_{600}) of each culture was standardized, and equal amounts of inoculum were inoculated with a inoculating needle into a test tube containing TY soft agar (0.3%) medium. The motility and spread of the strains grown in the soft agar tube was observed 4 days after inoculation at 28 °C.

2.5. Growth determination

Wild-type strain USDA 110 and the BDT-28pvo mutant (the *corA* gene encoding magnesium transporter was mutated) were cultured on TY medium at 28 °C to mid-log phase, and then were inoculated into liquid defined minimal medium (Krol and Becker, 2004) supplemented with either 0.01 mM (Mg^{2+}-limiting) or 1 mM (Mg^{2+}-sufficient) $MgSO_4$. The starting density (OD_{600}) of the culture was adjusted to 0.05. The growth was measured using spectrophotometer. The growth phenotype of BDT-2pvo mutant (*ppsA* gene encoding for phosphoenolpyruvate synthase was knocked out) was tested in same liquid defined minimal medium (Krol and Becker, 2004), but the carbon source (mannitol) was replaced by pyruvic acid. Growth was detected same to the above method using spectrophotometer.

3. Results

3.1. Characterization of the mutated genes

The inserted positions by Tn*5* transposons in the 14 genes in USDA 110 genome (GenBank No. NZ_CP011360) was shown in Table 1 and Fig. 1. The locus, length, deduced gene product and percentage of amino acid identity using BLASTP were shown in Table 1. The amino acid sequences of the annotated genes had identities ranging from 55 to 100% with those deduced from other bacteria (Table 1). Based on the highest identity of each sequence, we proposed and revised the functions of these 14 genes (Table 1). Among them, functions of three mutated genes in mutants BDT-4, BDT-5 and BDT-16 were unknown and were annotated as hypothetical membrane protein and hypothetical proteins. Another three mutants (BDT-18, −19 and −22) were